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Human *fur* Gene Encodes a Yeast KEX2-like Endoprotease That Cleaves Pro-β-NGF In Vivo

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Abstract. Extracts from BSC-40 cells infected with vaccinia recombinants expressing either the yeast *KEX2* prohormone endoprotease or a human structural homologue (*fur* gene product) contained an elevated level of a membrane-associated endoproteolytic activity that could cleave at pairs of basic amino acids (-LysArg- and -ArgArg-). The *fur*-directed activity (furin) shared many properties with Kex2p including activity at pH 7.3 and a requirement for calcium. By using antifurin antibodies, immunoblot analysis detected two furin translation products (90 and 96 kD), while immunofluorescence indicated localization to the Golgi apparatus. Coexpression of either Kex2p or furin with the mouse β-nerve growth factor precursor (pro-β-NGF) resulted in greatly enhanced conversion of the precursor to mature nerve growth factor. Thus, the sequence homology shared by furin and the yeast *KEX2* prohormone processing enzyme is reflected by significant functional homology both in vitro and in vivo.

POSTTRANSLATIONAL proteolysis is a common mechanism required for the synthesis of biologically active proteins and peptides in all eukaryotes examined, including yeast (21), invertebrates (56), and mammalian cells (17, 62). One of the early events in precursor protein maturation is endoproteolysis at the carboxyl side of pairs of basic amino acid sequences (especially -LysArg- and -ArgArg-). This type of endoproteolytic cleavage was initially inferred from the sequences of several endocrine and neuroendocrine precursor proteins and was first proposed from studies of proinsulin (9, 64) and the ACTH/β-endorphin precursor, proopiomelanocortin (POMC)[1] (11). Subsequent studies have revealed a broad spectrum of precursor proteins that require endoproteolysis at pairs of basic amino acids to yield mature peptides, including serum factors (3), viral polyproteins (43, 49, 51, 52), growth factors (24, 26, 58, 75), and receptors (76).

Several activities capable of cleaving at single or paired basic residues in vitro have been proposed as candidates for authentic mammalian precursor processing endoproteases (6, 10, 12–16, 25, 31, 32, 39–41, 46, 47). However, none of these candidate activities have been shown to be a bona fide precursor cleaving endoprotease in vivo. In contrast, genetic (33, 37) and biochemical (20, 22) studies unequivocally identified the gene required for excision of the peptide mating hormone (α-factor) from its precursor in *Saccharomyces cerevisiae* (baker's yeast) (for review see reference 21). This locus, the *KEX2* gene, encodes a unique subtilisin-like, membrane-bound, calcium-dependent, serine endoprotease (Kex2p) specific for cleaving on the carboxyl side of pairs of basic residues (-LysArg- and -ArgArg-) (22).

Several lines of evidence suggest that the mammalian precursor processing endoprotease(s) is functionally similar to Kex2p. The mammalian precursor proalbumin, for example, is efficiently converted in vitro to albumin by Kex2p, with cleavage at a paired basic amino acid sequence (-Arg-Arg-) (2). Furthermore, coexpression of the *KEX2* gene with mouse POMC in BSC-40 cells (a line incapable of processing this peptide precursor) resulted in efficient proteolysis, at pairs of basic amino acids, producing authentic pituitary peptides, including γ-LPH and β-endorphin 1-31 (68).

Recently, two human DNA sequences, *fur* and PC2, have been reported which share significant structural homology with the *KEX2* gene sequence (Fig. 1). The *fur* locus was initially identified by its proximity to the *fes/fps* proto-oncogene (53) and its homology to Kex2p was noted subsequently (23). The DNA sequence of the entire protein predicts a translation product, furin (72), which shares nearly 50% overall sequence identity with Kex2p in the 300-residue region comprising the catalytic domain, including the conserved active site Asp, His, and Ser. PC2 was identified by amplification of a human insulinoma library by the polymerase chain reaction using KEX2-derived primers and also shares a high degree of homology with Kex2p, especially in the active site domains (59). Both proteins show

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1. *Abbreviations used in this paper*: moi, multiplicity of infection; NGF, nerve growth factor; POMC, proopiomelanocortin; pro-β-NGF, β-nerve growth factor precursor; WGA, wheat germ agglutinin.

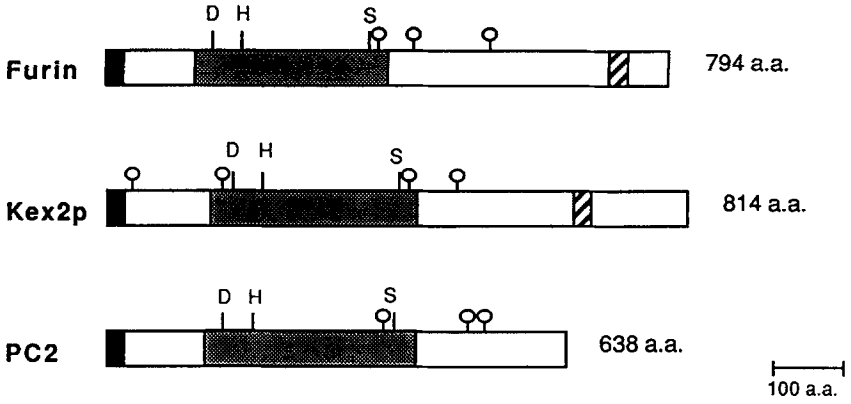

Figure 1. Schematic representation of furin, Kex2p, and PC2. The amino acids forming the catalytic triad in the active site are shown (*D*, aspartate; *H*, histidine; *S*, serine). Signal sequences are represented as solid black boxes and the subtilisin-like domains are shown as shaded regions. Hatched boxes indicate the putative transmembrane domains. (⚲), potential sites for attachment of asparagine-linked sugars. See references 72 (furin), 44 (KEX2), and 59 (PC2) for predicted protein sequences.

significantly greater homology to Kex2p than to bacterial subtilisins.

Aside from homology in their catalytic domains, the yeast *KEX2* gene and human furin cDNA also share very similar configurations (Fig. 1). Both proteins have a strongly hydrophobic NH_2-terminal sequence. In the case of Kex2p, this segment has been shown to target the protein to the secretory pathway, where it becomes glycosylated (20, 22, 23). Both proteins also possess a single highly hydrophobic segment near the COOH-terminus (44). In Kex2p, this region has been shown to be a transmembrane domain responsible for anchoring the enzyme in the secretory system, probably the Golgi apparatus (23, 48). Finally, both proteins contain highly charged COOH-terminal tails.

The sequence homology and shared topology suggested that the furin cDNA encodes a Kex2p-like protease that could be a functional human homologue of Kex2p. Here we demonstrate that indeed the furin cDNA encodes a Golgi-localized, membrane-associated, endoprotease that displays a substrate specificity, both in vitro and in vivo, similar to Kex2p. Possible functions of furin in mammalian cells are discussed.

Materials and Methods

Cell Culture

BSC-40, an African Green monkey kidney epithelial cell line and AtT-20, a mouse pituitary corticotroph cell line were cultured as described (68, 70). HepG2 cells, a human hepatoma, were cultured in MEM containing 10% heat inactivated fetal calf serum (HyClone Laboratories, Logan, UT) supplemented with 0.1 mM pyruvate and 0.1 mM nonessential amino acids (Gibco Laboratories, Grand Island, NY).

Vaccinia Virus

Vaccinia virus strain WR was used in these studies and infections were performed as described (68, 70). To construct the vaccinia recombinant expressing the human furin cDNA (VV:hFUR), plasmid pBluescript-PACE containing the human furin cDNA from which the 3′-untranslated region was deleted (74) was digested with Sca I and the insert excised by digestion with Hinc II and Sma I. Bgl II linkers were ligated on the 2.5-kb insert and the fragment was ligated into the vaccinia recombination plasmid, pVV3, which was previously cut with Bgl II. The orientation of the ligated cDNA was verified and the resulting plasmid pVV3:hFUR was used for marker transfer into vaccinia as previously described (29). To construct a vaccinia recombinant directing expression of the mouse β-nerve growth factor precursor (pro-β-NGF) cDNA (VV:mNGF), plasmid pGEM/ngf, containing a full-length mouse β-NGF cDNA (71) was digested with Hind III and incubated with Klenow polymerase to generate blunt ends. The linearized, blunt-ended fragment was then digested with Bam HI to yield an 820-bp insert coding for the shorter transcript of β-NGF (using the second initiation codon) (18). pVV3 plasmid was digested with Bgl II, incubated with T4 DNA polymerase to generate blunt ends, and digested with Bam HI. Vector and insert were then ligated and a plasmid containing the mNGF insert (pVV3:mNGF) was used to introduce the mouse NGF cDNA into vaccinia virus by standard marker transfer procedures (29).

Immunofluorescence

Cells grown on coverslips were rinsed in PBS (2.8 mM KCl, 1.5 mM KH_2PO_4, 1.4 mM NaCl, 8 mM Na_2HPO_4) with 1 mM $MgCl_2$ (PBS-M) and fixed for 15 min in PBS-M containing 3% paraformaldehyde plus 1 mM $CaCl_2$. After fixation, cells were permeabilized in 0.2% BSA, 0.1% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ in PBS. Next the fixed cells were preincubated in 0.1% TX-100 and 2% normal goat serum (NGS) to block nonspecific binding of antibodies. Coverslips were rinsed briefly and incubated overnight at 4°C with rabbit antifurin antiserum (1:50). The antifurin antibodies were generated against the catalytic domain of furin by expression in *Escherichia coli* of amino acids 146–372 of furin as a fusion to human superoxide dismutase (74). The furin antiserum was preabsorbed with an acetone precipitate of a VV:WT infected BSC-40 cell extract and incubated at room temperature for 4 h followed by clarification of the antiserum by centrifugation in a SPIN-X column (Costar, Cambridge, MA). After overnight incubation with the furin antiserum, coverslips were then rinsed in permeabilizing solution, incubated with a biotinylated goat anti-rabbit antiserum (1:200; Vector Laboratories, Inc., Burlingame, CA) for 30 minutes, rinsed and incubated with FITC-conjugated avidin (1:500, Vector Laboratories, Inc.) for 50 min. Coverslips were again rinsed and incubated with wheat germ agglutinin (WGA; diluted 1:50, Molecular Probes Inc., Eugene, OR) for 30 min at room temperature, rinsed, mounted on microscope slides in 50% glycerol, and viewed with a Leitz Dialux 22 EB fluorescent microscope (63× objective).

Cell Fractionation and In Vitro Proteolytic Assays

BSC-40 cells were grown to confluency on 100-mm plates in MEM containing 10% fetal calf serum. Parallel plates were infected with either VV:WT, VV:KEX2, or VV:hFUR at a multiplicity of infection (moi) of 5. At 18 h after infection, cells were chilled on ice, rinsed once in ice-cold PBS-M, harvested by scraping in 0.5 ml of 10 mM Hepes, pH 7.3, and lysed with 20–30 strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) (~70% total lysis as determined by trypan blue exclusion). Lysates were cleared by centrifugation at low speed (1,000 *g*, 5 min) to remove cellular debris and the crude extract was transferred to an ultracentrifuge tube. After centrifugation at 100,000 *g* for 60 min, the supernatant fraction was removed and the pellet was resuspended in 200 μl of 10 mM Hepes, pH 7.3. For assay, 25 μl was placed in 200 μl of sample buffer (100 mM Hepes, pH 7.3, 1% Triton X-100, 1 mM $CaCl_2$, 50 μM boc-gly-lys-arg-MCA [Peninsula Laboratories, Inc., Belmont, CA]). Samples were incubated for 30 min at 37°C and the quantity of liberated amino methyl coumarin was determined by fluorimetry (excitation = 380λ, emission = 460λ). Protein determinations were performed with bicinchoninic acid procedure (61).

Western Blot

For immunoblotting, samples containing equivalent amounts of total pro-

tein were loaded onto a 10% polyacrylamide gel and run in SDS buffer as originally described (54). Protein was then transferred under electric current (24 V) for 16 h at 4°C to nitrocellulose in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 15% methanol). The blot was preincubated in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Triton X-100, 0.02% NaN_3) containing 3% dried nonfat milk for 50 min at room temperature followed by incubation at room temperature with primary antibody (rabbit antifurin, diluted 1:8,000) for 3 h. After washing three times with TBST, the blot was incubated with goat anti-rabbit-alkaline phosphatase and developed (Promega ProtoBlot Immunoblotting system, Promega Corp., Madison, WI).

Northern Blot

All RNA manipulations were performed as described (54) and as presented in the legend to Fig. 4.

Immunoprecipitations

Cells were grown to confluency in MEM containing 10% fetal calf serum in 35-mm, 6-well plates. Parallel wells were infected with VV:NGF (moi = 2) and either VV:WT, VV:KEX2 or VV:hFUR (moi = 5, total moi = 7). After 18 h, cells were starved for 30 min in MEM (-Met, -Cys) + 10% dialyzed fetal calf serum, then pulsed for 3 h with [^{35}S]Met,Cys (100 μCi/well; NEN Express Label, DuPont Co., Wilmington, DE). All further treatment of samples was at 4°C. Medium was harvested and centrifuged for 5 min at 1,500 *g*, and transferred to a fresh tube. Samples were then centrifuged at 10,000 *g* for 20 min and transferred to a fresh tube. A pool of four anti-β-NGF rat monoclonal antibodies generated to 2.5S NGF was added to each sample and incubated for 2 h. A second antibody (rabbit anti-rat IgG + M; Zymed Laboratories Inc., South San Francisco, CA) was added and samples were incubated for 1 h. Finally, protein A-Sepharose was added to each sample and incubated for 1 h. The Sepharose beads were washed two times with RIPA buffer (1% NP-40, 1% SDS, 1% sodium deoxycholate [DOC], 150 mM NaCl, 50 mM Tris-HCl, pH 8) and two times with NET-N (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8, 0.5% NP-40, 0.1% SDS, 0.01% NaN_3), and then loaded on 12.5% Laemmli-type SDS polyacrylamide gel.

Results

To express a functional furin translation product in mammalian cells, the human furin cDNA, isolated from a hepatoma (HepG2) cDNA library (74), was inserted into a vaccinia virus expression vector by standard methods (29). Plates of BSC-40 cells were infected either with wild-type vaccinia virus (VV:WT), with a vaccinia recombinant containing the yeast *KEX2* gene (VV:KEX2), or with a vaccinia recombinant containing the furin cDNA (VV:hFUR). Cells were harvested 20 h after infection and crude membrane fractions were prepared. Each sample was analyzed for proteolytic activity capable of cleaving fluorogenic peptide substrates containing a pair of basic amino acids. Endoproteolytic activity capable of cleaving substrates containing LysArg- (Table I) increased 4.9- and 2.3-fold, respectively, in cells infected with VV:KEX2 or VV:hFUR. A similar stimulation was found for cleavage of substrates containing -ArgArg- (data not shown). The increased proteolytic activity observed in either VV:KEX2 or VV:hFUR infected cells was quantitatively associated with the 100,000-*g* membrane pellet. This selective partitioning to cellular membranes is consistent with earlier observations for Kex2p in yeast (20, 22, 45) and for Kex2p expressed in BSC-40 cells (68).

In addition to its apparent substrate specificity, furin shares other enzymic properties with Kex2p. Kex2p is a serine protease that is active at neutral pH, strictly calcium dependent, and inhibited by only high concentrations of PMSF (22, 45; Table I). Similarly, the furin-dependent activity was inhibited by EGTA (a calcium chelator) and was active at pH

Table I. Expression of Kex2p and Furin Activity in Mammalian Cells

		Condition		
	Virus	2 mM Ca^{++}	+3 mM EGTA	2 mM Ca^{++} +1 mM PMSF
HSS	WT	12	32	17
	KEX2	56	15	29
	FUR	18	35	24
HSP	WT	109	131	45
	KEX2	533	137	295
	FUR	250	110	64
Media	WT	16	27	21
	KEX2	25	22	24
	FUR	21	23	23

BSC-40 cells were grown to confluency on 100-mm plates in MEM containing 10% fetal calf serum. Parallel plates were infected with either VV:WT, VV:KEX2 or VV:hFUR (moi = 5). At 18 h after infection crude membrane fractions were prepared and proteolytic activity was determined using a small fluorogenic substrate (boc-Gly-Lys-Arg-MCA) as described in Materials and Methods. Values are listed as enzyme units per milligram protein. One enzyme unit equals 1 mol of substrate converted per minute. Values shown are from a single experiment, but qualitatively similar results were obtained in three completely independent trials. *HSS*, high speed supernatant; *HSP*, high speed pellet.

7.3 (Table I). Unlike the yeast prohormone endoprotease, however, furin activity appeared to be much more sensitive to PMSF. Neither Kex2p nor furin activity were detected in the culture medium (Table I).

Aliquots of the supernatant and pellet fractions from the samples described in Table I, in addition to control samples from mock-infected cells, were resolved by one-dimensional SDS gel electrophoresis and furin translation products were identified by immunoblotting with specific antibodies (Fig. 2). A doublet of proteins with the apparent sizes of 90 and 96 kD, respectively, was detected and was found only in the membrane fraction from VV:hFUR infected BSC-40 cells (Fig. 2, lane *6*). These apparent molecular masses are in good agreement with the 87-kD translation product predicted by the furin cDNA. No furin was detectable by this method

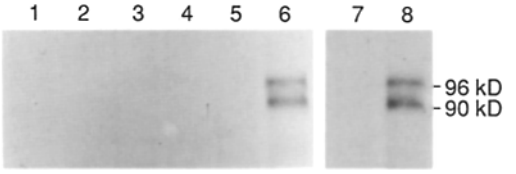

Figure 2. Immunodetection of furin. High speed supernatant (lanes *1*, *3*, and *5*) and pellet (lanes *2*, *4*, and *6*) fractions from BSC-40 cells either mock-infected (lanes *1* and *2*) or infected with VV:WT (lanes *3* and *4*) or VV:hFUR (lanes *5* and *6*) were prepared as described in Table I. PAGE and immunoblotting were prepared as described in Materials and Methods. In a separate experiment the high speed pellet prepared from VV:hFUR-infected BSC-40 cells in the presence of 1 mM PMSF and 2 μg/ml leupeptin was resuspended by Dounce homogenization in 0.1 M sodium carbonate (pH 11) followed by recentrifugation at 100,000 *g*. Proteins from the high speed carbonate supernatant (lane *7*) and pellet (lane *8*) fractions were precipitated with TCA, resolved by gel electrophoresis, furin protein was identified by Western blot analysis as described in Materials and Methods.

in the soluble fractions from these cells or in samples from either mock- or VV:WT infected BSC-40 cells (Fig. 2, lanes *1–5*).

To establish whether furin is tightly associated with membranes, the 100,000-*g* pellet prepared in the presence of 1 mM PMSF and 2 μg/ml leupeptin from BSC-40 cells infected with VV:hFUR was extracted with 0.1 M sodium carbonate (pH 11) and resedimented at 100,000 *g*. Western analysis demonstrated that the furin doublet was retained in the pellet fraction (Fig. 2, compare lanes *7* and *8*), as expected for the behavior of an integral membrane protein.

Because Kex2p-dependent precursor cleavage appears to occur in the Golgi apparatus in yeast (34), immunofluorescence microscopy was used to determine the subcellular localization of furin in mammalian cells. Plates of BSC-40 cells were infected with either VV:WT or VV:hFUR and the fixed cells were stained with antifurin antiserum. Cells infected with VV:hFUR revealed intense perinuclear staining (Fig. 3 *A*). No detectable staining was evident in control cells infected with VV:WT (Fig. 3 *C*). The fixed cells were also incubated with rhodamine-coupled wheat germ agglutinin (WGA), a specific Golgi compartment marker (28, 73). As shown in Fig. 3 *B*, the pattern of staining by WGA was superimposable with the localization of furin in BSC-40 cells. Thus, as predicted for Kex2p in yeast, furin appears to be a Golgi compartment-associated enzyme in mammalian cells.

The structural (Fig. 1) and enzymic properties (Table I) shared by the yeast *KEX2* endoprotease and furin suggested that this novel human endoprotease may be responsible for processing precursors containing paired basic sites to produce mature bioactive products in the secretory pathway.

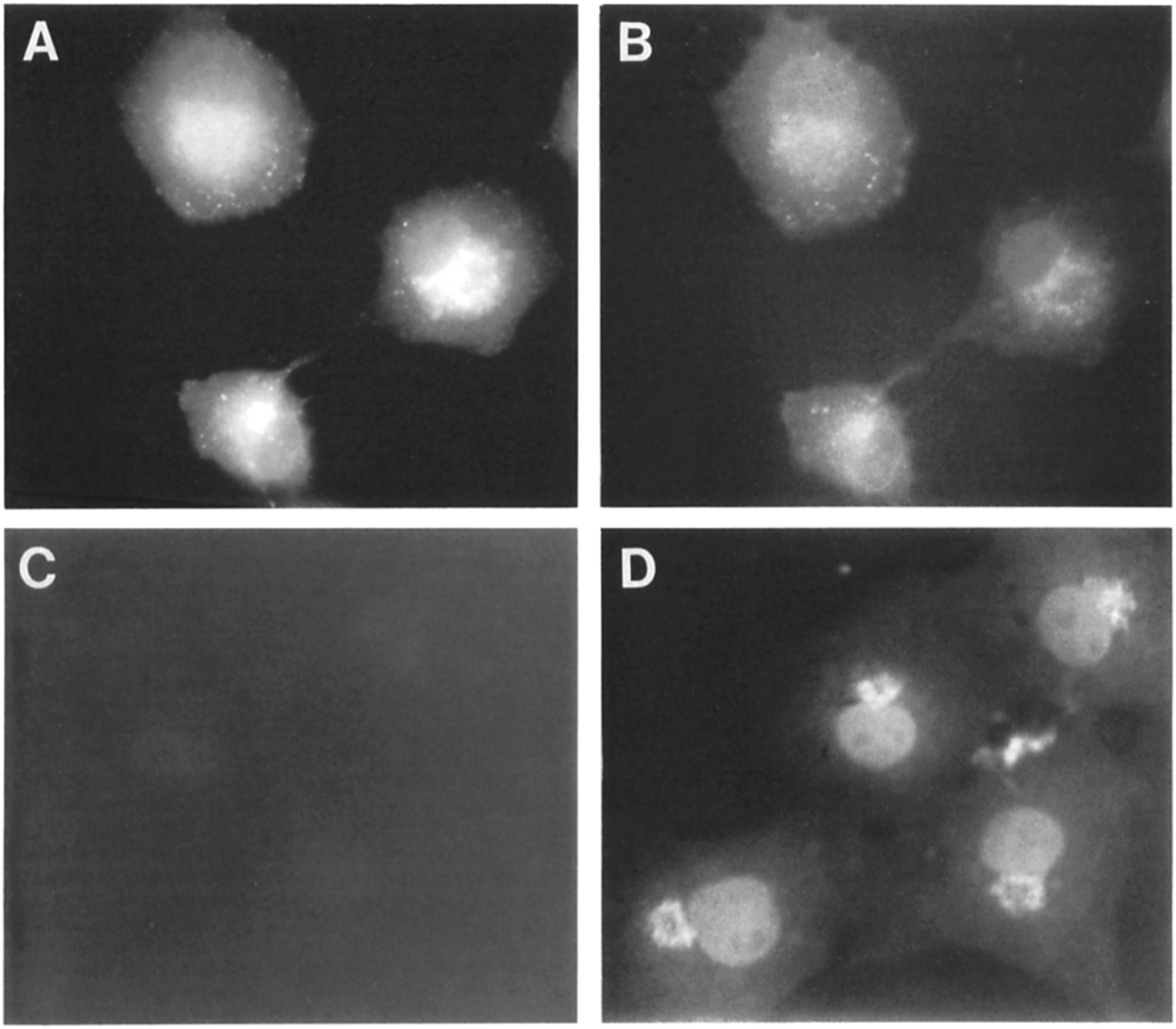

Figure 3. Immunolocalization of furin. Parallel cultures of BSC-40 cells grown on coverslips were infected with either VV:hFUR (*A* and *B*) or VV:WT (*C* and *D*) at an moi of 1. AFter 18 h, the cells were fixed in 3% paraformaldehyde and processed for immunofluorescence microscopy as described in Materials and Methods. (*A* and *C*). Fluorescein fluorescence (furin antiserum). (*B* and *D*). Rhodamine fluorescence (WGA binding).

However, a wide variety of precursor proteins, synthesized in a large number of endocrine and nonendocrine tissues, require endoproteolysis at cleavage sites composed of a doublet of basic residues. To facilitate identification of authentic furin substrates, we examined the cell type specificity of furin gene expression. Our initial screening of cell lines indicated detectable levels of furin transcript in all cell types examined including AtT-20 (mouse pituitary corticotroph), BSC-40 (African Green monkey kidney epithelial), GH4C1 (rat pituitary somato/lactotroph), HeLa (human cervical epithelial), HepG2 (human hepatoma), L (mouse fibroblast), NG108-15 (rat glioma × mouse neuroblastoma hybrid), and Rin m5F (rat insulinoma). Fig. 4 shows a comparison of relative furin transcript levels in three of these cell lines including the cell line used as a source for the cloned cDNA (HepG2) and two cell lines (AtT-20 and BSC-40) previously characterized for endogenous precursor protein processing capability (67–70). As expected, a 4.5-kb furin transcript was detected in poly A+ RNA from HepG2 cells, the human hepatoma cell line from which the furin cDNA was isolated (74) (Fig. 4, lane *C*). This cell line endogenously expresses and processes proalbumin at an -ArgArg- site (1, 36). Only very low levels of furin transcript were detected in the mouse pituitary corticotroph line, AtT-20 (Fig. 4, lane *A*). These cells express high levels of POMC and efficiently process this precursor in the regulated secretory pathway (27) at several sites composed of paired basic amino acids (60). Note that a second 3.3-kb RNA band, detected only with the AtT-20 RNA, hybridized to the radiolabeled furin probe. Interestingly, this species is approximately the size of PC2 mRNA (59). Whether this band represents PC2, a different furin transcript, or yet another *KEX2* homologue expressed in endocrine cells remains to be determined. The highest levels of furin transcript were found in BSC-40 cells, a monkey kidney epithelial line (Fig. 4, lane *B*). These cells possess only a constitutive secretory pathway (70) and are incapable of processing certain neuroendocrine peptide precursors (including POMC and proenkephalin [67–70]. However, BSC-40 cells endogenously express and process the transforming growth factor β precursor (pro-TGF-β) at [illegible]ls (24). Furthermore, when expressed in BSC-40 cells, the nerve growth factor precursor (pro-β-NGF) was cleaved at a basic residue doublet (Lys^{119}-Arg^{120}, Fig. 5 *A*) to produce mature β-NGF (19). One potential role, therefore, of the furin endoprotease in mammalian cells is to process growth factor precursors (as opposed to neuropeptide precursors).

A vaccinia virus recombinant that directs expression of the mouse pro-β-NGF cDNA (VV:mNGF) was constructed to study the processing of this precursor protein by furin in the mammalian cell secretory pathway. BSC-40 cells were selected since this cell line was used to document vaccinia-derived furin expression (Table I; Figs. 2 and 3), possesses only the constitutive secretory pathway (70) and is amenable to infection with multiple vaccinia recombinants (68).

Replicate plates of BSC-40 cells were infected with either VV:WT or VV:mNGF or both, and at increasing times after virus infection each culture was metabolically labeled for 3 h with [^{35}S]methionine. The secreted products were immunoprecipitated with pooled monoclonal antibodies directed against β-NGF and resolved by SDS gel electrophoresis. In agreement with earlier studies (19), metabolic labeling of BSC-40 cells at short times after infection (3 h) resulted in expression of pro-β-NGF and efficient processing of the prohormone to mature 13-kD β-NGF peptide, presumably by

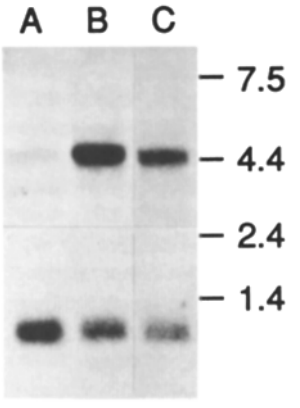

Figure 4. Analysis of furin mRNA. 5 μg of poly A$^+$ RNA isolated from AtT-20 (*A*), BSC-40 (*B*), and HepG2 (*C*) cells was denatured with formaldehyde, subjected to electrophoresis in a 1% agarose gel, transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL), and hybridized with a random-prime ^{32}P-labeled insert containing the hfur cDNA sequence. After exposure to x-ray film, the blot was stripped and rehybridized with a random-primed ^{32}P-labeled probe of the 1B15 (cyclophilin) sequence to ensure that equal levels of RNA were loaded initially. hfurin mRNA migrates at 4.5 kb. 1B15 migrates at 1 kb.

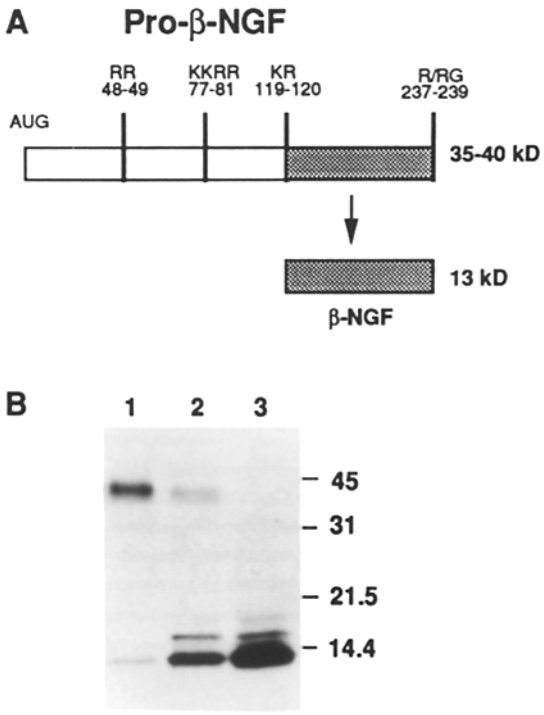

Figure 5. Processing of pro-β-NGF by Kex2p and furin in vivo. (*A*) Schematic of pro-β-NGF expressed from VV:mNGF vector (14), with cleavage sites at paired basic amino acids (*vertical bars*) and mature β-NGF. (*B*) Immunoprecipitation of β-NGF from the medium of BSC-40 cells infected with (*1*) VV:WT + VV:mNGF, (*2*) VV:KEX2 + VV:mNGF, or (*3*) VV:hFUR + VV:mNGF. Immunoprecipitations were performed as described in Materials and Methods using an equal volume of media from equal amounts of cells. Pro-β-NGF migrates as a 35–40-kD band, whereas mature β-NGF migrates as a 13-kD band. Processing intermediates appear at 18 and 20 kD.

the endogenous furin enzyme present. However, when labeling was initiated at a later time (19-h postinfection), the rate of pro-β-NGF synthesis was significantly higher and the major secreted product was unprocessed precursor (Fig. 5 *B*, lane *1*). This observation provided a means to examine the effect of elevated furin protease activity on the efficiency of processing of pro-β-NGF.

Either Kex2p or furin was coexpressed with pro-β-NGF in BSC-40 cells. 18 h after infection, each sample was incubated for 3 h with [^{35}S]methionine and β-NGF-containing peptides were immunoprecipitated from the culture media using pooled monoclonal antibodies directed against β-NGF. Under these conditions, coexpression of Kex2p together with pro-β-NGF markedly increased processing of pro-β-NGF to mature (13 kD) β-NGF peptide (Fig. 5 *B*, lane *2*). In addition, two peptides of 18 and 20 kD were also immunoprecipitated. These peptides probably represent processing intermediates resulting from cleavage within the proregion of the precursor (32). Thus, Kex2p, which can efficiently process mouse POMC in mammalian cell (68), also can process pro-β-NGF to β-NGF peptide. Strikingly, coexpression of human furin and pro-β-NGF resulted in complete conversion of pro-β-NGF to smaller peptides, primarily 13-kD β-NGF (Fig. 5 *B*, lane *3*). As a control to ensure that the apparent pro-β-NGF processing was not occurring in the medium after secretion, labeled pro-β-NGF from the culture medium of VV:mNGF-infected cells was transferred to plates of either VV:WT- or VV:hFUR-infected cells. Analysis of this material after 3 h of incubation demonstrated that pro-β-NGF was completely stable in the culture medium.

Comparison of the 13-kD β-NGF to 35-kD pro-β-NGF by densitometric analysis revealed that in the sample from cells coinfected with VV:mNGF and VV:WT, only 23% of the radioactivity was present as 13-kD β-NGF; in marked contrast in cells coinfected with VV:mNGF and VV:KEX2 or VV:hFUR, the fraction of radioactivity present as 13-kD β-NGF was 79 and >98%, respectively. The processed NGF in the medium from cells coinfected with VV:mNGF and VV:hFUR was biologically active as determined by its ability to induce neurite outgrowth on chick dorsal root ganglia, whereas medium from cells infected with VV:WT alone was negative for this biologic activity (Bresnahan, P., and G. Thomas, unpublished results). Because the vaccinia-produced furin activity measured by in vitro assay of the infected cells was substantially lower than vaccinia produced Kex2p activity (Table I), yet pro-β-NGF conversion was complete in the furin producing cells but not in the Kex2p-producing cells, the human endoprotease appears to be a more efficient processing enzyme for this precursor in BSC-40 cells.

Discussion

Our results demonstrate that the human furin cDNA isolated from HepG2 cells encodes a Golgi compartment-localized membrane-associated, calcium-dependent, serine protease. In agreement with the reported structural homology between furin and the yeast *KEX2* prohormone endoprotease (23, 72), we show that both enzymes share significant functional homology in vitro (Table I) and in vivo (Fig. 5).

The Western blot analysis in Fig. 2 revealed two prominent furin translation products (90 and 96 kD) present in crude membrane fractions of VV:hFUR-infected BSC-40 cells. The cause of the size heterogeneity is not yet known. One possibility is that the doublet may result from heterogeneity in posttranslational modifications (e.g., note in Fig. 1 the multiple sites for attachment of asparagine-linked sugars). However, the doublet is still present after treatment with N-glycanase (74). A second possibility is that the initial furin translation product is actually an inactive zymogen which becomes activated by endoproteolytic cleavage. Indeed, autoproteolytic removal of the entire amino terminal extension is required for activation of the structurally related bacterial subtilisin precursor (30, 50). Similarly, the precursor of Kex2p protease undergoes removal of an NH_2-terminal propeptide, probably by an autoproteolytic mechanism (Wilcox, C. A., and R. S. Fuller, personal communication). The furin cDNA sequence predicts a small cluster of paired basic amino acids at nearly the identical position as found in *KEX2*. Cleavage at either of these sequences would generate a protein ~6 kD smaller than the primary translation product (the same size difference detected in Fig. 2). The observed doublet is not likely to be merely an artifact of sample preparation because it was found when harvesting in the presence of protease inhibitors (Fig. 2) or when the cells were extracted directly with buffer containing 2% SDS at 70°C (Leduc, R., and G. Thomas, unpublished results).

The preferential localization of furin to the Golgi apparatus of BSC-40 cells (Fig. 3) suggests conserved mechanisms for intracellular targeting of processing proteases between yeast and humans. Intracellular retention of Kex2p in the Golgi apparatus of *Saccharomyces cerevisiae* is required for efficient maturation of α-mating factor. Yeast mutants lacking clathrin heavy chain are unable to retain Kex2p intracellularly, resulting in deficient processing of the pro-α-mating pheromone (48). Similarly, deletions of the Kex2p cytoplasmic tail also result in mislocalization of the protease and inefficient processing of the pro-α-mating pheromone (23). The observation that both Kex2p and furin share a highly charged cytoplasmic tail, including conserved tyrosine residues (23, 72), suggests that the furin cytoplasmic tail may also be required for retention of this protease in the Golgi compartment. Golgi compartment localization of furin in our experiments is unlikely to be an artifact of overproduction of the protein because several other proteins of known subcellular localization reach their proper destination when produced from vaccinia expression vectors. For example, in polarized epithelial cells, VSV-G protein and influenza virus HA antigen are correctly targeted to the basolateral and apical membranes, respectively, when expressed by vaccinia virus recombinants (65). Likewise, addition of the KDEL endoplasmic retention signal onto a soluble form of CD4 expressed from vaccinia confers retention of this protein to the ER (7). The actual compartment in which furin-directed proteolysis occurs remains to be determined. However, the apparent localization to the Golgi compartment and substrate specificity of this endoprotease correlates with the reported endoproteolysis of several retroviral envelope proteins (including RSV Pr95 [49] and HIV gp160 [63]), which apparently requires a -LysArg- directed endoprotease and takes place in the Golgi apparatus.

Processing of peptide precursors at pairs of basic residues has been studied most extensively in endocrine cell lines

where efficient processing of neuroendocrine prohormones is restricted to the regulated secretory pathway (8, 27, 35). When missorted into the constitutive secretory pathway, prohormone molecules are not processed, but secreted as the intact precursor (27, 70). However, processing of other precursor proteins at doublets of basic residues occurs in cells that lack the regulated secretory pathway (e.g., BSC-40, L, HeLa) including pro-β-NGF (19; Fig. 5), pro-TGF-β (58), proalbumin (1, 36), pro–von Willebrand factor (5, 74), insulin proreceptor (76), and several retroviral envelope proteins (e.g., HIV gp160) (43, 49, 51, 52). The widespread tissue distribution of this large class of precursor proteins suggests that the cognate endoprotease(s) must also be present in a wide variety of tissue and cell types. Consistent with these findings, furin mRNA is found ubiquitously in all cultured cell lines tested, although markedly different levels of expression are observed (Fig. 4; and Thomas, L., and G. Thomas, unpublished results).

While both prohormone (e.g., POMC) and other precursors (e.g., pro-β-NGF) require proteolysis at paired basic amino acids to liberate biologically active polypeptides, the dependence of prohormone processing on localization to the regulated secretory pathway suggests a specific mechanism for maturation of this class of substrate. This restriction may arise from: (*a*) structural differences in the precursor molecules, including sequences in the vicinity of the processing site or higher order structural properties (e.g., folding); (*b*) differential compartmentalization of the substrate and the processing enzyme(s); or, perhaps, (*c*) expression of distinct processing enzymes in the regulated pathway. One notable structural similarity shared by several "nonhormone" substrates, including the precursors for factor IX (3), insulin receptor (76), endothelin (75), TGF-β (58), EGF (26), β-NGF (4), brain-derived neurotropic factor (BDNF) (38), neurotrophin 3 (42), and HIV gp160 (43), is an arginine located four residues amino-terminal to the paired basic cleavage site (-*Arg*-X-Lys/Arg-Arg-). The lack of processing of a mutant pro-factor IX (*Arg*-Pro-Lys-Arg-→ -*Gln*-Pro-Lys-Arg-) suggests that this residue is required (3). In contrast, the neuroendocrine prohormone, prosomatostatin, is not processed in fibroblasts (57) even though it possesses an arginine residue four amino acids amino-terminal to the paired basic cleavage site. However, the apparent processing site on prosomatostatin is an -Arg-Lys- sequence, not -LysArg- or -ArgArg- typically found in nonhormone precursors. Mutation of this -ArgLys- site in prosomatostatin to -ArgArg- or -LysArg- results in a partial processing of this prohormone in fibroblast cells (66) suggesting that -ArgArg- or -LysArg- are indeed the preferred sites for the processing enzyme in the constitutive pathway, presumably furin.

Consistent with this view, using pro-β-NGF as a model substrate, we demonstrated that furin expressed in a recombinant vaccinia vector efficiently processed a mammalian precursor protein in the constitutive secretory pathway of BSC-40 cells. Taken together, the results presented here unequivocally identify furin as the initial member of a unique family of mammalian proteases capable of processing precursor proteins in the secretory pathway.

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